Hair as a long-term retrospective cortisol calendar in orang-utans (Pongo spp.): New perspectives for stress monitoring in captive management and conservation

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Hair as a long-term retrospective cortisol calendar in orang-utans (*Pongo spp.*): New perspectives for stress monitoring in captive management and conservation

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Abstract

This study examined whether the method of hair cortisol analysis is applicable to orang-utans (*Pongo spp.*) and can help to advance the objective monitoring of stress in non-human primates. Specifically, we examined whether fundamental prerequisites for hair cortisol analysis are given in orang-utans and, subsequently, whether segmental hair analysis may provide a retrospective calendar of long-term cortisol levels. For this, hair samples were examined from 71 zoo-living orang-utans (38 males, 33 females, mean age = 23.5 years) for which detailed records of past living conditions were available. Hair samples were cut from defined body regions and were analyzed either in full length or in segments. Results showed that hair cortisol concentrations (HCC) were unrelated to age or sex of the individual animal. HCC were found to be higher in orang-utans, with perceived long-term stressful periods (mean HCC = 43.6 ± 26.5 pg/mg, n = 13) compared to animals without perceived stressful periods (19.3 ± 5.5 pg/mg, n = 55, $P < 0.001$). In non-stressed animals, segmental hair analyses revealed that HCC was stable along the hair shaft even when hair reached >40 cm. The possibility of obtaining a retrospective calendar of stress-related cortisol changes through hair analysis was further supported by data of three case studies showing close correspondence between the segmental HCC results and keeper reports of stress exposure during the respective time periods. Finally, low within-animal variation in HCC from different body regions (CV%: 14.3) suggested that this method may also be applicable to naturally shed hair, e.g., as found in nests of wild orang-utans and other great apes. Therefore, using HCC may provide an ideal non-invasive tool for both captive management as well as conservation in orang-utans and potentially other great apes.

1 Introduction

Until recently, it has been rather difficult to assess the endocrine consequences of chronic stress in animals such as non-human primates. The traditionally used non-invasive cortisol assessment methods in urine (Bahr et al., 2000; Hauser et al. 2008), faeces (Weingrill et al., 2011) or saliva (Fuchs et al., 1997) reflect cortisol secretion during a narrow time window. Therefore, measuring long-term hormone levels requires repeated sampling of the same individual in order to even out the influence of short-term stressful events and/or biological cycles. Such a procedure, however, requires animals to be kept in captivity or wild animals to be well habituated to humans which may limit the ecological validity of the respective data. Furthermore, locating wild animals on a regular basis might be difficult in certain species and terrain even if animals are habituated, and habituation is not always desirable because it is time-consuming (Bertolani and Boesch, 2008) and may expose animals to threats of poaching (Morgan and Sanz, 2003).

Measuring cortisol in hair now opens new possibilities for the study of long-term biological consequences of chronic stress exposure. Davenport et al. (2006) was the first to present evidence that the cortisol concentration in hair of rhesus macaques reflects the integrated stress-induced activity of the hypothalamic-pituitary-adrenal axis during hair growth. Since then, hair cortisol measurement has received increasing attention in an ever growing number of fields of application, in both humans (see Stalder and Kirschbaum, 2012) and a variety of animal species (e.g., horses: Anielski, 2011; polar bears: Bechshøft et al. 2011, 2012; cows: Comin et al., 2008; rabbits: Comin et al., 2012; non-human primates: Fourie and Bernstein, 2011). Besides providing a long-term endocrine record, one key advantage of hair is the stability of HCC under ambient keeping conditions. This

enables easy storage and posting of samples (see Stalder and Kirschbaum, 2012), which could make this method highly valuable in remote places.

Given the continuous growth of hair, an important additional benefit is its potential to derive a retrospective cortisol calendar from segmental hair analyses. However, this is still highly debated in human research. Various studies have suggested that the clinical course of patients with pathological hyper- or hypocortisolism appears to be well represented in their segmental HCC profile (Manenschijn et al., 2011; Thomson et al., 2010), but other studies have not confirmed this (D'Anna-Hernandez et al., 2011; Kirschbaum et al., 2009). To use hair as a retrospective cortisol calendar a number of biological preconditions must be met: First, cortisol incorporation into the hair matrix must be largely completed before the hair reaches the skin surface. Later incorporation (e. g. through sweat) is a potential influence on cortisol levels in human scalp hair (Russell et al., 2012, Skoluda et al., 2012) or ungulate hair (Anielski, 2008, Bullard et al., 1970), but is unlikely to influence HCC in non-human primates because sweat glands are mainly inactive and mostly restricted to their forehead, palms and armpits (Montagna, 1972). Second, there must be stability of HCC over time. Thus, the systematic decrease in cortisol along the hair shaft seen in humans ('washout effect'; e.g., Kirschbaum et al., 2009) should not be observed. Hamel et al. (2011) have shown a decrease in HCC in the hair of rhesus macaques after numerous intense wash/dry procedures using shampoo or water only. Zoo-living animals, however, are not subject to frequent rain and thus a washout effect is unlikely to affect hair of captive animals. Supporting this, no studies have not shown cortisol washout effect in animal hair, either in captive (Davenport et al., 2006) or free-ranging animals (Fourie and Bernstein, 2011, Bechshøft et al., 2011, Bechshøft et al., 2012). Third, growth rates of individual hairs within the hair strand should be uniform and the majority of hairs must be in the same growth phase. For cut or pulled-out hair, this means that most hairs should to be in the

anagen (active growing) phase for the correlation between segments and time periods to hold. By contrast, naturally shed hairs found in sleeping nests of wild great apes (Goossens et al., 2006; Nater et al., 2011), are mainly in their telogen (quiescent) phase (Jeffery et al., 2007). Courtois et al. (1995) found that human hairs were shed two to three months after the beginning of the telogen phase. Thus, hairs shedding naturally at the same time should represent roughly the same time window. This may also apply to orang-utan hairs and would thus allow segmental analysis of a bundle of shed hairs. However, nest hair originates from various, unknown body regions. It is therefore important to confirm that the incorporation of cortisol into hair is constant within and across body regions and that HCC is not influenced by local blood circulation or other unknown body region-specific factors.

In order to evaluate the utility of hair cortisol analysis for the use in captive and wild-living orang-utans (*Pongo spp.*), the current study set out to provide a careful examination of several fundamental prerequisites. Specifically, we examined (I) the influence of sex and age on mean HCC, (II) the stability of cortisol concentrations in orang-utan hair over the whole hair shaft to control for systematic cortisol leaching over time and external contamination, and (III) whether body region had a significant influence on HCC in orang-utans. To assess the feasibility of using segmental hair analysis on an individual level, we furthermore investigated (IV) the hair growth rate in orang-utans to enable assignment of specific hair segments to their corresponding time window. Finally, to validate hair cortisol analysis in orang-utans, we examined whether (V) highly stressful periods were retrospectively reflected in corresponding hair segments over a prolonged period of time on an individual case level.

2 Methods

2.1 Animals

Samples were collected from a total number of 71 captive orang-utans (38 males, 33 females) from 26 European zoos (males: mean age = 22 years, range = 1 - 54 years; females: mean age = 24, range 4 - 52 years). Keepers and curators filled out questionnaires for all individuals, including information on age, sex, group composition, ranking, weight (if possible) and periods that were assumed to be stressful for animals during the last two years. The latter periods included major changes in group composition with intra-group conflicts, transfer and severe/chronic illness. Based on these subjective keeper reports, animals with perceived stressful periods were defined as 'stressed' animals (*n = 15)* whereas those without perceived stressful periods were defined as 'non-stressed' animals (*n* = 56).

2.2 Sample collection and preparation

Hair growth rate was assessed from three animals aged 29 (male), 26 (female) and 1 (male) by shaving and re-shaving of the same patch 4-6 weeks later. Growth rate was estimated as the regrown hair length divided by the number of days following shaving. For practical reasons, hair growth rates were obtained from different body regions.

For 62 animals, hair samples were cut approximately 1 cm above the skin (up to 8 samples from different body regions per individual). For 9 additional animals, hairs were collected from sleeping sites, resulting in a total number of 71 animals. To test for the general stability of HCC along the hair shaft, hair samples of 18 non-stressed animals (random body regions) with at least 15 cm long hair were cut into segments of 3 cm prior to analysis.

Furthermore, all hair samples with a sufficient amount of material and at least 9 cm length were cut into segments of 3 cm in order to examine whether time-limited stressors resulted in higher variation of HCC across segments (animals with time-limited stressors: *n* = 10; animals with stable living conditions: *n* = 29).

In addition, samples of three individuals met the criteria for segmental hair analysis with temporal assignment. These individuals provided hair of at least 9 cm length and furthermore had experienced severe stressful periods of at least one month. Samples including at least 100 single hairs per strand were segmented into 2 or 3 cm. Subsequently, each segment was analysed as described in 2.3 and HCC was plotted against the individual corresponding timeline.

To examine potential differences in HCC between different body regions we examined hair samples from six defined regions. For practical reasons, we included all animals which provided samples from three (*n* = 5), four (*n* = 1), five (*n* = 6) and six (*n* = 5) of the defined body regions. This resulted in a total number of 78 samples from 17 animals in this part of the study (right wrist upside: *n* = 12, left wrist upside: *n* = 12, stomach: *n* = 14, central back: *n* = 11, right shoulder: *n* = 15, left shoulder: *n* = 15). HCC of each hair strand was measured over the full length of hair. However, when different hair strands of the same animal showed considerable variation in length, longer strands of hairs were adjusted in length to match the shortest strands. Therefore, the examined time window could differ between individuals but was homogeneous within individuals.

2.3 Hair cortisol analysis

For hair cortisol analysis, a slightly modified protocol from Stalder et al. (2012, part study II) was followed. Samples were washed twice with 3 ml of isopropanol and dried over night. For hormone extraction, a strand of at least 100 hairs was minced into 3-5 mm

pieces in order to increase the stability of results (Fourie, 2012). 10 mg of this pool were incubated with 1.8 ml of methanol for 18 hours at 45°C. Subsequently, 1 ml of the extract was dried and resuspended in 400 µl phosphate buffer. Cortisol concentrations were determined using a commercially available immunoassay with chemiluminescence detection (CLIA, IBL-Hamburg, Germany). Intra- and inter-assay coefficients of variation of this assay are below 8%.

2.4 Statistical analysis

Hair cortisol data were not found to be normally distributed. Logarithmic transformations most effectively reduced the skewing of distributions and were applied for inferential analyses. For descriptive purposes, information on mean values and standard deviations are presented in original units (pg/mg). Three animals died without signs of sickness at old age (> 49 years) within ten months after hair sampling. All of them were males and exhibited markedly increased hair cortisol values of at least two standard deviations above the mean of non-stressed individuals. As the underlying long-term endocrine mechanisms during that stage of life are largely unknown, these individuals were excluded from subsequent analyses in order to avoid false positives. For the between-subject examination on general effects of sex, age and perceived long-term stress (> 1 month) on HCC, individual hair cortisol levels were calculated as a mean of all available hair samples for each animal. A Pearson correlation was run to examine the relationship between age and HCC. To identify effects of sex and perceived stress on HCC, a 2 x 2 analysis of variance (ANOVA; male vs. female and stressed vs. non-stressed) was conducted. Because juveniles still enjoy a high degree of freedom in their behaviour the respective effects were mainly expected in adults. Therefore, juveniles (< 10 years, Weingrill et al., 2011) were excluded from this particular analysis.

187 A repeated-measures ANOVA was used to test for differences in HCC across hair
188 segments. As this analysis aimed to study the stability of HCC under stable living
189 conditions, only hair samples of animals were included that had no perceived major
190 stressful periods over the examined period.

191 To investigate whether unstable living conditions with time-limited stressors (1-3
192 months) resulted in a generally increased HCC variation between segments, a two-tailed
193 Student *t*-test was conducted comparing animals living in stable conditions with those
194 animals with time-limited stressors. In this analysis, the coefficient of variation (CV%)
195 across different hair segments was used as dependent variable.

196 The comparison of HCC between different body regions was conducted using a
197 repeated-measures ANOVA with Greenhouse-Geisser corrections being applied to
198 account for violation of sphericity. Because repeated-measures ANOVA would discard
199 incomplete sets of data, we increased the statistical power for this analysis by replacing
200 missing HCC values of individual body regions (n = 23 of 102) by use of a multiple
201 imputation as recommended by others (Rubin, 2009, Schafer and Graham, 2002). All
202 statistical analyses were conducted with SPSS for windows, version 19 (IBM, Chicago, IL).

203 ## 3 Results

204 ### 3.1 Influence of sex, age and stress on HCC

205 The mean ± SD HCC values of all examined individuals were 28 ± 18.6 pg/mg (range:
206 9 to 108 pg/mg; males: 28 ± 18.6; females: 20 ± 6.4 pg/mg; juveniles: 19 ± 7.6 pg/mg). The
207 mean ± SD HCC of the males that died of old age were 70.3 ± 32.7 pg/mg (range: 40 to
208 105 pg/mg) and those data were excluded from subsequent analyses. HCC was found to
209 be unrelated to age (r = 0.12, P = 0.34, n = 68). The two-way (stress x sex) ANOVA

revealed a main effect of stress ($F_{(1, 64)}$ = 23.92, P < 0.001, η^2_p = 0.27; see Figure 1), illustrating significantly higher HCC in stressed animals (43.6 ± 26.5 pg/mg, n = 13) than in non-stressed animals (19.3 ± 5.5 pg/mg, n = 55). No main effect of sex ($F_{(1,64)}$ = 3.7, P = 0.06, η^2_p = 0.06) or a stress x sex interaction ($F_{(1,64)}$ = 0.4, P = 0.52, η^2_p = 0.01) were found.

3.2 Cortisol stability and variability along hair shaft

In non-stressed animals with hair strands of 15 cm, no differences in HCC were found between the five consecutive 3 cm hair segments ($F_{(2.6, 44.7)}$ = 1.6, P = 0.2, n = 18; see Figure 2). In line with this, the examination of HCC in a single animal with very long hair (42 cm representing ~3.5 years) also revealed a very stable HCC profile across all 14 segments (35.2 ± 2.5 mg/pg). Furthermore, the HCC between segments varied significantly more in hair samples of animals for which keepers had reported some stressful periods (CV% = 32.8 ± 16.5%, n = 10) than in animals which had lived under stable conditions (CV% = 16.1 ± 9%, n = 29; $t_{(37)}$ = 3.79, P < 0.001).

3.3 Influence of body regions on HCC

HCC did not differ between the six defined body regions ($F_{(2.4, 38.1)}$ = 1.10, P = 0.4, n = 17 animals). Figure 3 shows for each of the six defined body regions the mean percentage deviation from the animal's mean HCC, where in each comparison the mean HCC was based on all other body regions except the one being compared (right wrist: -6 %; left wrist: -7 %; stomach: 0 %; back: 1 %; right shoulder: 6 %; left shoulder: 7 %). For all 28 animals of which hair samples from two or more body regions were available, the mean CV% of HCC between body regions was 14.3% (range = 4.7% to 29.6%).

3.4 Hair as a retrospective endocrine calendar – individual case reports

The mean growth rate was found to be 0.98 cm / 30 days (individual 1: 1.1 ± 0.13 cm / 30 days; individual 2: 0.89 ± 0.18 cm / 30 days; individual 3: 0.95 ± 0.16 cm / 30 days; see Figure 4c).

Based on the assessed hair growth rate, figure 4a (Individual A) shows the hair cortisol profile of a 44-year old female that was frequently attacked by another female group member. Physical aggression toward A increased and culminated in 2009. The keepers therefore repeatedly changed group composition in order to handle the situation. Plotting the hair cortisol profile against the time axis revealed 4-5 fold elevated HCC compared to non-stressed animals (86 pg/mg) in times of exposure to direct aggression in February-April 2010 (i.e. serious biting, chasing, food stealing) and 2-3 times elevated cortisol levels (53 pg/mg) in times of exposure to indirect or psychological aggression in August-October 2010 (permanent contact to main aggressor through iron bars, behavioural displays of the main aggressor). On the other hand, HCC dropped to average levels in a period of total isolation from other orang-utans or during reintegration to the group after removal of the main aggressor.

Figure 4b illustrates the HCC profile of individual B, a 29-year-old female orang-utan that, by coincidence, used to be the main aggressor of A. The cortisol profile of B retrospectively revealed that HCC was elevated 3-4 fold above the average level of non-stressed animals (75 pg/mg) during times of total isolation (May-June 2010) and an operation (removal of uterus: July 2010). However HCC remained at a high level when B was reintegrated into the group with indirect contact through iron sliders with A. HCC levels even increased up to 5 fold over the average values (103 pg/mg) after the transfer to another zoo in October 2010, but finally decreased over the following nine months when the animal increasingly habituated to the new environment.

Figure 4c shows the hair cortisol profile of a one-year-old baby orang-utan. His mother died of chronic airsacculitis when he was eight months old (February 2011). The orphan was adopted by another female of the group about two weeks after the mother's death (March 2011). After the adoption, HCC reduced from 3-fold the average values (64 pg/mg) to levels of non-stressed animals (28 pg/mg).

4 Discussion

The current study on orang-utans shows that the analysis of the stress-responsive hormone cortisol in hair is a highly valuable tool, which may advance long-term stress monitoring. Specifically, the current results support the notion that a retrospective calendar of cumulative cortisol secretion may be derived from segmental hair analyses across the whole length of hair. This was supported by the finding that there is no HCC decrease over time in captive orang-utans and that HCC in proximal to distal segments remains stable even when hair reached lengths of more than 40 cm. This is in accordance with previous studies on HCC in animals (rhesus macaques: Davenport et al., 2006; grizzly bears: Macbeth et al., 2010) but contrasts with the leaching effect that was found towards distal hair segments in some research on human hair (D'Anna-Hernandez et al., 2011; Gao et al., 2010; Kirschbaum et al., 2009). Further studies may be warranted to investigate the effects of frequent rain on the concentration of hair cortisol in free-ranging orang-utans as indicated by first results of *in vitro* research on pooled hair samples from rhesus macaques (Hamel et al., 2011).

Equally important were our findings of discrete HCC peaks in hair segments corresponding to the time of perceived stress. Previously, only a small number of studies on human scalp hair suggested that segmental hair analyses reflected at least 1.5 years of the clinical course of patients treated for pathological hyper- or hypocortisolism

(Manenschijn et al., 2011; 2012; Thomson et al., 2010). The only other attempt to provide a retrospective cortisol calendar from segmental hair analysis in animals was made in horses. However, this trial failed, which was potentially due to external contamination through sweat (Anielski, 2008). The cortisol profiles of our three orang-utan case studies are therefore the first that show the feasibility of segmental hair analysis in animals. This reflects several key points. First, cortisol molecules are mainly incorporated into the matrix of orang-utan hair during or shortly after the formation and second, the molecules do not move along the hair shaft after the incorporation, unlike cocaine molecules (Henderson et al., 1996). Third, the clear cortisol peaks in distant segments indicate that neighbouring hairs grow at the same rate, and therefore, reflect the same time window. Finally, the present study shows that in captive orang-utans the length of the retrospective cortisol calendar is only dependent on the length of hair, with longer hairs reflecting longer time windows.

The orang-utans have long hair, but working with short hair requires particular attention. Samples of shorter hair will contain a smaller percentage of hair in the anagen (growth) phase and an increased percentage of telogen, non-growing hair (Courtois et al. 1995). Because anagen and telogen hair vary in their corresponding time window, one segment consisting of multiple anagen and telogen hairs, integrates different time windows within a single segment. Therefore, segments of neighbouring hairs are unlikely to represent the same time window in short haired animals or body regions. In order to still enable the application of segmental hair analysis to shorter hair, we suggest that the longest (telogen) hair could be removed prior to the analysis.

This is the first study to show the feasibility of segmental hair analysis using non-human primate hair. One strand of approximately 100 hairs enabled us to track the cortisol level of individuals for up to several years, given an adequate length of hair. The observed results highlight the potential of this method for the application in animal welfare to objectively judge keeping conditions, group composition and translocation success. However, in order to fully apply segmental hair analysis, more research on hair growth is necessary. To our knowledge, the present study provides the first data on hair growth rates in orang-utans. There is limited information on hair growth rates in other primate species (Fourie, 2012). Our results indicate that hair growth rates in all three tested orang-utans were similar to that in humans. In contrast, Fourie (2012) reported that rates in two gorillas (*Gorilla gorilla gorilla)* varied from 1.2 cm/month to 5.8 cm/month. However, we belief this degree of variation on growth rates in the same species is unlikely and is potentially due to methodological issues.

The baseline cortisol levels indicated that sex did not appear to have significant influence on HCC. This is in line with results from faecal glucocorticoid in orang-utans (Weingrill et al., 2011) as well as in human hair (Stalder et al., 2013; but see Dettenborn et al., 2012). Furthermore, our results did not show any age-related HCC pattern. Very few other studies investigated the effect of age on the baseline cortisol levels in non-human primates. Weingrill et al. (2011) found slightly increased faecal glucocorticoid concentrations in older individuals. Similar to our findings, Sapolsky and Altmann (1991) generally found no influence of age on cortisol concentration in yellow baboons but described hypercortisolism in the oldest individuals (> 16 years). In contrast, Fourie and Bernstein, (2011) described infant hypercortisolism in vervet monkeys and guinea baboons. However, due to missing samples of small infant orang-utans (<1 year) our data does not allow any conclusion on this matter.

Our results suggest that body region did not have a significant influence on HCC in orang-utans. This is an important precondition for the application of hair cortisol measurement in wild orang-utans which requires the use of naturally shed hair found in sleeping nests (Goossens et al., 2006; Jeffery et al., 2007; Nater et al., 2011). Besides representing a different time window than cut (anagen) hair, shed hair is likely to originate from different body regions. Our results on the influence of body region on HCC are in accordance with results found in rabbits (Comin et al., 2012) but contrast with one animal study that showed significantly higher HCC in the neck region in grizzly bears (Macbeth et al., 2010). However, further studies are required to find out if this reflects a species-specific process. Most importantly for the application of hair cortisol measurement in wild orang-utans, the coefficient of variation for different samples of the same animal was comparatively low. This will allow the use of shed hair which is a mix of different body regions.

In conclusion, this study shows that orang-utan hair can be used as a long-term retrospective cortisol calendar for stress monitoring. Therefore, segmental hair cortisol analysis is a powerful tool for captive management. Furthermore, our results encourage the use of naturally shed hair to assess basal cortisol concentrations. Because shed hair in nests can be obtained non-invasively and even from animals which are not habituated this will open new possibilities in field endocrinology and conservation.

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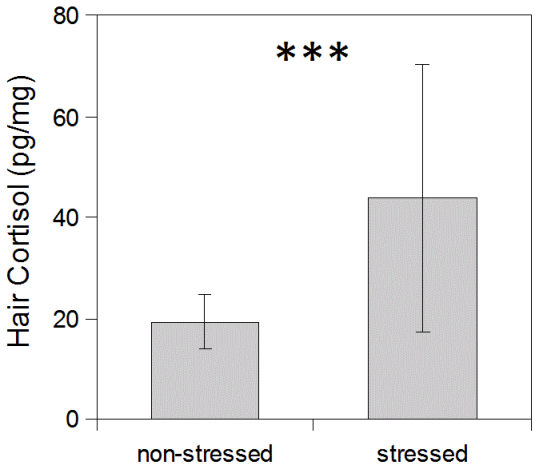

Figure 1: Mean hair cortisol concentration (with standard deviation) of orang-utans without perceived stressful periods ('non-stressed': n = 55) and with perceived stressful periods ('stressed': n = 13).

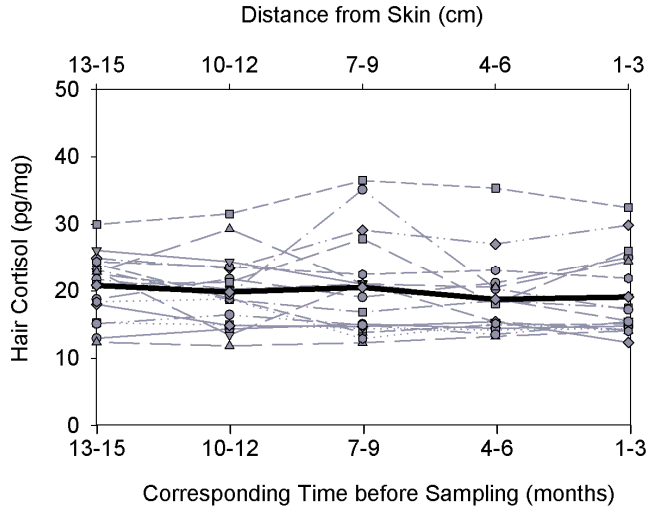

Figure 2: Hair cortisol profiles of individual orang-utans with a hair length ≥ 15 cm (n = 18). HCC are shown for five consecutive segments of 3 cm. Each data point represents a mean HCC of about 3 months. Mean values of all 18 profiles are illustrated by a solid black line.

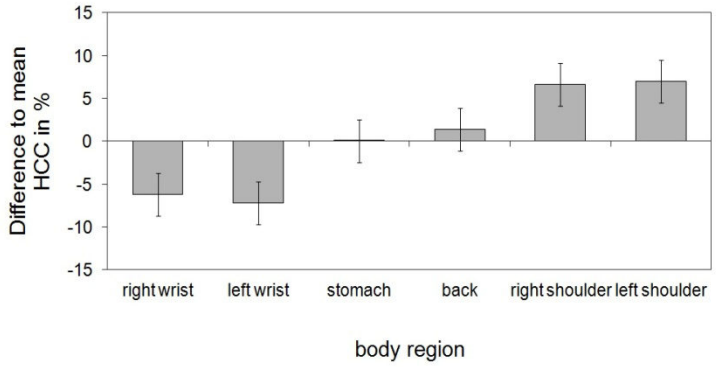

Figure 3: Percentage difference (with standard error) between defined body regions and the mean hair cortisol concentration (HCC, excluding the respective body region). There was no significant difference between body regions. Values are based on a total of 79 samples from 17 orang-utans: n (right wrist) = 12, n (left wrist) = 12, n (stomach) = 14, n (back) = 11, n (right shoulder) = 15, n (left shoulder) = 15.

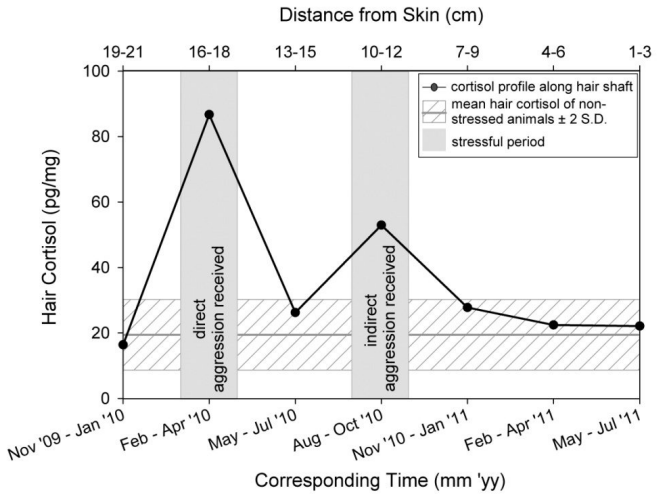

Figure 4a: Cortisol profile along a strand of hairs of the female orang-utan A. Each data point represents the mean cortisol concentration of a three months period. Estimated individual hair growth rate = 1.0 cm / month.

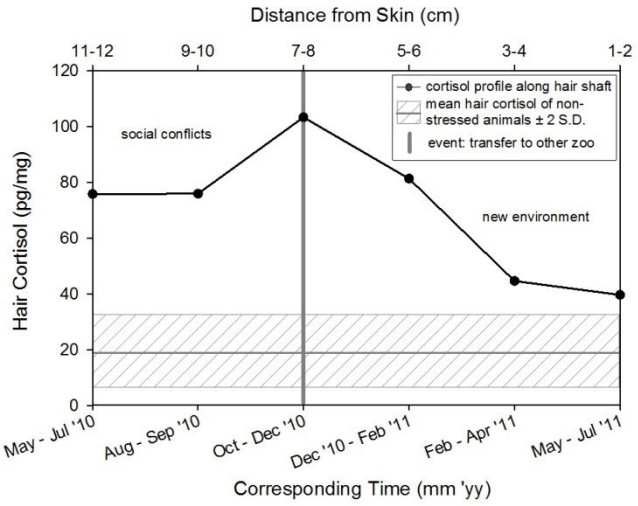

Figure 4b: Cortisol profile along a strand of hairs of the female orang-utan B. Each data point represents the mean cortisol concentration of a two months period. Estimated individual hair growth rate = 0.9 cm / month.

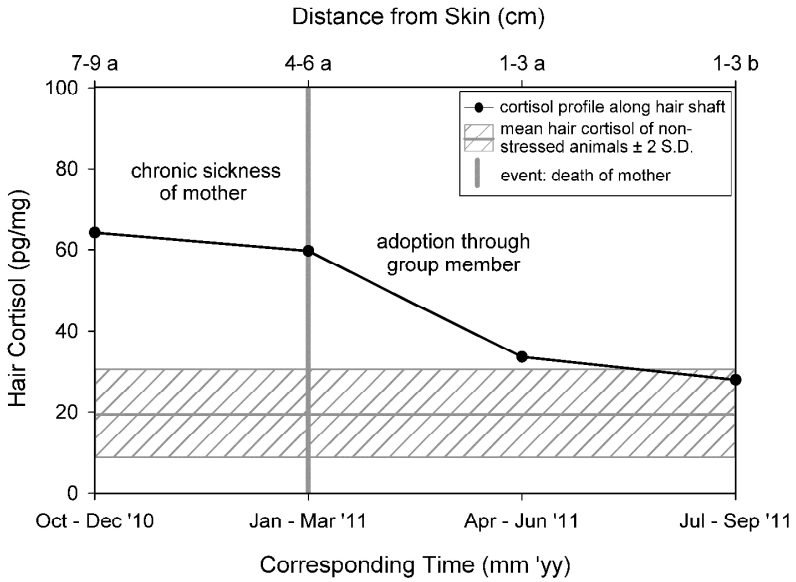

Figure 4c: Cortisol profile of two strands of hairs of a one-year old orphaned baby orang-utan (hair strand b was cut three months after a). Each data point represents the mean cortisol concentration of a three months period. Measured individual hair growth rate = 0.95 cm / month.